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the documents annexed hereto are true copies of:

Application forms P.1 and P.3, provisional specification and  
drawings of South African Patent Application No. 2002/4977  
as originally filed in the Republic of South Africa on 20  
June 2002 in the name of DUNEAS, Nicolaas  
for an invention entitled: "OSTEOINDUCTIVE BIOMATERIALS".

Geteken te  
Signed at

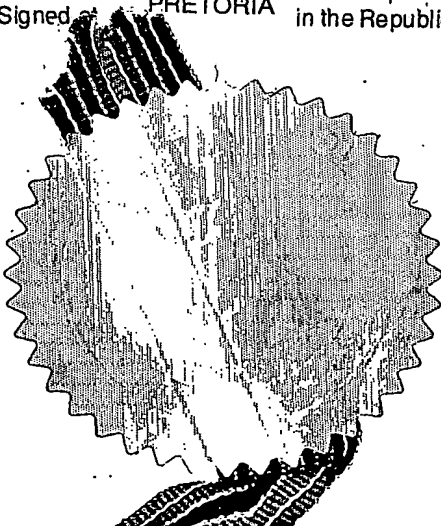
PRETORIA

In die Republiek van Suid-Afrika, hierdie  
in the Republic of South Africa, this

30th

dag van  
day of

June 2003



Registrateur van Patente

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REPUBLIC OF SOUTH AFRICA  
PATENTS ACT, 1978  
APPLICATION FOR A PATENT AND  
ACKNOWLEDGEMENT OF RECEIPT  
(Section 30(1) Regulation 22)

FORM P.1  
(to be lodged in duplicate)

20.6.02

060001

THE GRANT OF A PATENT IS HEREBY REQUESTED BY THE UNDERMENTIONED APPLICANT  
ON THE BASIS OF THE PRESENT APPLICATION FILED IN DUPLICATE

21 01 PATENT APPLICATION NO 2002/000001 A&A REF V15270

71 FULL NAME(S) OF APPLICANT(S)

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54 TITLE OF INVENTION

OSTEOINDUCTIVE BIOMATERIALS

Only the items marked with an "X" in the blocks below are applicable.

☐ THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. The earliest priority claimed is

Country:

No:

Date:

☐ THE APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO 21 01

☐ THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON  
APPLICATION NO 21 01

THIS APPLICATION IS ACCOMPANIED BY:

- ☒ A single copy of a provisional specification of 23 pages  
☒ Drawings of 2 sheets  
☐ Publication particulars and abstract (Form P.8 in duplicate) (for complete only)  
☐ A copy of Figure of the drawings (if any) for the abstract (for complete only)  
☐ An assignment of invention  
☐ Certified priority document(s). (State quantity)  
☐ Translation of the priority document(s)  
☐ An assignment of priority rights  
☐ A copy of Form P.2 and the specification of RSA Patent Application No 21 01  
☒ Form P.2 in duplicate  
☒ A declaration and power of attorney on Form P.3  
☐ Request for ante-dating on Form P.4  
☐ Request for classification on Form P.9  
☐ Request for delay of acceptance on Form P.4  
☐ Extra copy of informal drawings (for complete only)

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Dated this 20 day of June 2002

ADAMS & ADAMS  
APPLICANTS PATENT ATTORNEYS

The duplicate will be returned to the applicant's address for service as  
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REPUBLIC OF SOUTH AFRICA  
PATENTS ACT, 1978  
DECLARATION AND POWER OF ATTORNEY  
(Section 30 - Regulation 8, 22(i)(c) and 33)

|                       |                 |                 |              |              |
|-----------------------|-----------------|-----------------|--------------|--------------|
| PATENT APPLICATION NO |                 | A&A Ref: V15270 | LODGING DATE |              |
| 21                    | 06/06/2002/4977 |                 | 22           | 20 JUNE 2002 |

|                              |                  |
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| 72                          | DUNEAS, Nicolaas |

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|---------------------------|---------|--------|------|-----|----|-----|
| EARLIEST PRIORITY CLAIMED | COUNTRY | NUMBER | DATE |     |    |     |
|                           | 33      | NIL    | 31   | NIL | 32 | NIL |

NOTE: The country must be indicated by its International Abbreviation - see schedule 4 of the Regulations

|                    |                              |
|--------------------|------------------------------|
| TITLE OF INVENTION |                              |
| 54                 | OSTEOCONDUCTIVE BIOMATERIALS |

\* I/We Nicolaas Duneas

hereby declare that :-

1. I/we am/are the applicant(s) mentioned above;
- ~~\*\*\* 2. I/we have been authorized by the applicant(s) to make this declaration and have knowledge of the facts herein stated in the capacity of \_\_\_\_\_ of the applicant(s);~~
- \*\*\* 3. the inventor(s) of the abovementioned invention is/are the person(s) named above and the applicant(s) has/have acquired the right to apply by virtue of an assignment from the inventor(s);
4. to the best of my/our knowledge and belief, if a patent is granted on the application, there will be no lawful ground for the revocation of the patent;
- ~~\*\*\*\* 5. this is a convention application and the earliest application from which priority is claimed as set out above is the first application in a convention country in respect of the invention claimed in any of the claims; and~~
6. the partners and qualified staff of the firm of ADAMS & ADAMS, patent attorneys, are authorised, jointly and severally, with powers of substitution and revocation, to represent the applicant(s) in this application and to be the address for service of the applicant(s) while the application is pending and after a patent has been granted on the application.

SIGNED THIS 19<sup>th</sup> DAY OF JUNE 2002

Full Names: Nicolaas Duneas

(no legalization necessary)

- \* In the case of application in the name of a company, partnership or firm, give full names of signatory/signatories, delete paragraph 1, and enter capacity of each signatory in paragraph 2.  
\*\* If the applicant is a natural person, delete paragraph 2.  
\*\*\* If the right to apply is not by virtue of an assignment from the inventor(s), delete "an assignment from the inventor(s)" and give details of acquisition of right.  
\*\*\*\* For non-convention applications, delete paragraph 5.

A & A Ref No: V15270

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FORM P6

REPUBLIC OF SOUTH AFRICA  
Patents Act, 1978

## PROVISIONAL SPECIFICATION

(Section 30 (1) - Regulation 27)

|    |    |                         |
|----|----|-------------------------|
| 21 | 01 | OFFICIAL APPLICATION NO |
|----|----|-------------------------|

2002/4977

|    |              |
|----|--------------|
| 22 | LODGING DATE |
|----|--------------|

20 June 2002

|    |                              |
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| 54 | TITLE OF INVENTION |
|----|--------------------|

OSTEOINDUCTIVE BIOMATERIALS

THIS INVENTION relates to osteoinductive biomaterials. In particular the invention relates to an osteogenic composition and to the use of an osteogenic composition in therapy.

The osteogenic composition of the invention is particularly intended for human or mammalian tissue regeneration and for promoting or inducing bone growth. For the purposes of this specification, the phrase "osteogenic protein" refers to the material which is obtained by fractionation of total mammalian bone protein and which is capable of inducing bone formation. The terms "osteogenic" and "osteoinductive" are considered to be synonymous. Osteogenesis is the term used to describe the *de novo* formation of bone in adult mammals and is evidenced in adults during regeneration of bone fractures. It proceeds *via* a process which closely resembles embryonic osteogenesis. Osteogenic protein contains, amongst other unidentified proteins, Bone Morphogenetic Proteins (BMPs). This is a family of characterized proteins which have been classified as part of the larger transforming growth factor-beta superfamily of morphogenic proteins. The BMP family comprises more than a dozen individual members which are known to be capable of inducing bone formation in mammals.

According to a first aspect of the invention, in a method of isolating osteogenic protein from bone, in which an osteogenic protein containing fraction is extracted from bone and enriched by a sequence of enrichment steps selected from

ultrafiltration and chromatography, there is provided the improvement of removing higher molecular weight components from the osteogenic protein containing fraction prior to the enrichment steps.

5           The higher molecular weight components may have a molecular weight of about 100 - 300 kDa. The higher molecular weight components will typically include collagen, collagen fragments and collagen aggregates.

10           The method may include removing the higher molecular weight components by ultrafiltration. For example, the components may be removed by ultrafiltration through a 100 - 300 kDa nominal molecular weight membrane such as a 100 - 300 kDa nominal molecular weight polysulphone membrane.

15           The osteogenic protein containing fraction may be extracted from the bone using a chaotropic solution. The chaotropic solution may contain urea, guanidinium chloride or combinations thereof.

20           The enrichment steps may include successive ultra-filtration of the osteogenic protein containing fraction through progressively smaller nominal molecular weight membranes followed by, or interspersed with, chromatographic enrichment steps.

25           The osteogenic protein containing fraction may be concentrated and desalted through 10 kDa and 5kDa ultra-filtration steps.

          The chromatographic enrichment steps may be selected from one or more of heparin-sepharose chromatography, hydroxyapatite chromatography and reverse-phase silica chromatography.

30           According to a second aspect of the invention, there is provided a bone growth inducing composition which includes osteogenic protein, insoluble collagenous bone matrix (ICBM) and gelatin.

The composition may be in the form of a hydratable powder .

5 The insoluble collagenous bone matrix may be prepared by demineralization of whole bone powder with acid to produce an acid demineralised whole bone powder or matrix, extraction of soluble components from the demineralised bone powder or matrix with a chaotropic agent such as aqueous urea or a guanidinium solution, water-washing the residue and then drying the residue to produce insoluble collagenous bone matrix.

10 The gelatin may be obtained by boiling insoluble collagenous bone matrix in purified water to extract a fraction rich in soluble collagen type I, precipitating the material with ethanol and drying the precipitate *in vacuo*.

15 The insoluble collagenous bone matrix may be mammalian. It is preferably human insoluble collagenous bone matrix or hICBM. The gelatin may accordingly be human gelatin.

The osteogenic protein may be prepared by a method as hereinbefore described.

20 The bone growth inducing composition may include the osteogenic protein in an amount of about 400 - 600 $\mu$ g, the hICBM in an amount of about 800 - 1200mg and the human gelatin in an amount of about 100 - 1000mg. In a preferred embodiment, the bone growth inducing composition includes the  
25 osteogenic protein in an amount of about 500 $\mu$ g, the hICBM in an amount of about 1000mg and the human gelatin in an amount of about 200mg.

30 According to another aspect of the invention, there is provided a method of preparing a bone growth inducing composition, the method including the steps of combining osteogenic protein, insoluble collagenous bone matrix and gelatin.

The insoluble collageneous bone matrix may be mammalian and is preferably human insoluble collageneous bone matrix or hICBM. The gelatin may be human gelatin.

5 The osteogenic protein may be prepared by a method as hereinbefore described

10 The method may include combining the osteogenic protein in an amount of about 400 - 600 $\mu$ g, the hICBM in an amount of about 800 - 1200mg and the human gelatin in an amount of about 100 - 1000mg. In a preferred embodiment, the method may include combining the osteogenic protein in an amount of about 500 $\mu$ g, the hICBM in an amount of about 1000mg and the human gelatin in an amount of about 1200mg.

15 The method may include combining the osteogenic protein with the hICBM in a dilute aqueous acidic solution, lyophilising the resulting mixture to produce a dry powder and mixing the powder with the human gelatin to produce a hydratable material.

20 According to another aspect of the invention, there is provided a device for inducing bone growth in a mammal, the device including a bone growth inducing composition which comprises osteogenic protein, insoluble collagenous bone matrix and gelatin and a delivery mechanism for delivery of the composition to a treatment site.

25 The osteogenic protein, the insoluble collagenous bone matrix and the gelatin may be as hereinbefore described. The delivery mechanism may be a syringe.

30 In particular, the composition may be the hydratable powder hereinbefore described which may be contained in the syringe. Thus, by drawing an aqueous

saline solution up into the syringe, the material may be hydrated for injection into the delivery site.

5 According to another aspect of the invention, there is provided a method of inducing bone formation in a mammal having a skeletal defect, the method including the step of implanting a bone inducing composition as hereinbefore described into the skeletal defect of the mammal.

10 According to another aspect of the invention, there is provided a method of inducing the growth of ectopic bone in a mammal, the method including the step of implanting a bone inducing composition as hereinbefore described in a non-bony site of the mammal.

15 According to another aspect of the invention, there is provided a method of accelerating allogenic bone healing in a mammal, the method including the step of implanting allogenic bone material together with a bone inducing composition as hereinbefore described into site in which allogenic bone healing in the mammal is required.

20 The allogenic bone material may be tissue-banked bone including human cortical bone chips, cancellous bone blocks, cancellous bone powder, whole morselised bone or demineralised bone matrix.

25 According to another aspect of the invention, there is provided a method of accelerating autogenous bone graft healing in a mammal, the method including the step of implanting autogenous bone material together with a bone inducing composition as hereinbefore described into site in which autogenous bone graft healing in the mammal is required.

30 The autogenous bone material may be morselised iliac crest autogenous bone.

According to another aspect of the invention, there is provided a substance or composition for use in a method of inducing bone formation in a mammal having a skeletal defect, the substance or composition comprising a bone inducing composition as hereinbefore described and the method including implanting the composition into a skeletal defect of the mammal.

According to another aspect of the invention, there is provided a substance or composition for use in a method of inducing the growth of ectopic bone in a mammal, the substance or composition comprising a bone inducing composition as hereinbefore described and the method including the step of implanting a bone inducing composition as hereinbefore described in a non-bony site of the mammal.

According to another aspect of the invention, there is provided a substance or composition for accelerating allogenic bone healing in a mammal, the substance or composition comprising a bone inducing composition as hereinbefore described and the method including the step of implanting allogenic bone material together with a bone inducing composition as hereinbefore described into a site in which allogenic bone healing in the mammal is required.

The allogenic bone material may be tissue-banked bone including human cortical bone chips, cancellous bone blocks, cancellous bone powder, whole morselised bone or demineralised bone matrix.

According to another aspect of the invention, there is provided a substance or composition for accelerating autogenous bone graft healing in a mammal, the substance or composition comprising a bone inducing composition as hereinbefore described and the method including the step of implanting autogenous bone material together with a bone inducing composition as hereinbefore described into a site in which autogenous bone graft healing in the mammal is required.

The autogenous bone material may be morselised iliac crest autogenous bone.

5 According to another aspect of the invention, there is provided the use of a substance or composition in the preparation of a medicament for use in a method of inducing bone formation in a mammal having a skeletal defect, the substance or composition comprising a bone inducing composition as hereinbefore described.

10 According to another aspect of the invention, there is provided the use of a substance or composition for the preparation of a medicament for use in a method of inducing the growth of ectopic bone in a mammal, the substance or composition comprising a bone inducing composition as hereinbefore described.

15 According to another aspect of the invention, there is provided the use of a substance or composition in the preparation of a medicament for accelerating allogenic bone healing in a mammal, the substance or composition comprising a bone inducing composition as hereinbefore described and an allogenic bone material.

20 The allogenic bone material may be tissue-banked bone including human cortical bone chips, cancellous bone blocks, cancellous bone powder, whole morselised bone or demineralised bone matrix.

25 According to another aspect of the invention, there is provided the use of a substance or composition in the preparation of a medicament for accelerating autogenous bone graft healing in a mammal, the substance or composition comprising a bone inducing composition as hereinbefore described and an autogenous bone material.

30 The autogenous bone material may be be mercerised iliac crest autogenous bone.

This invention accordingly relates to the preparation of osteogenic protein from mammalian bone and to its use in conjunction with a collagenous matrix in bone repair.

### DISCUSSION

Mammalian bone tissue is host to a family of protein growth and differentiation factors, called the bone morphogenetic proteins (BMPs). These proteins are capable of inducing new bone formation when implanted in adolescent and adult mammals.

The BMPs are redeployed in adults to cause regeneration of bone *via* mechanisms closely resembling embryonic differentiation. The developmental cascade of bone differentiation consists of chemotaxis of mesenchymal cells, proliferation of progenitor cells, differentiation of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi, (1981) Collagen Rel. Res. 1:209-226). It has been shown that the natural endochondral bone differentiation activity of bone matrix can be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone inductive activity (Sampath and Reddi, (1981) Proc. Natl. Acad. Sci. USA 78:7599-7603). The purification of osteogenin, an osteogenic protein from mammalian bone is disclosed by Sampath et al. (1987) (Proc. Natl. Acad. Sci. USA 84, 7109 - 7113. Urist et al. (Proc. Natl. Acad. Sci. USA (1984) 81:371-375) disclose a bovine bone morphogenetic protein extract having the properties of an acidic polypeptide and a molecular weight of approximately 18 kD. The authors report that the protein is present in a fraction separated by hydroxyapatite chromatography, and that it induces bone formation in mouse hindquarter muscle and bone regeneration in trephine defects in rat and dog skulls.

European Patent Application No. 148,155, published Oct. 7, 1985, discloses osteogenic proteins derived from bovine, porcine, and human origin. One of the proteins, designated by the inventors as a P3 protein and having a molecular weight of 22-24 kD, is reported to have been purified to an essentially

homogeneous state. This material is reported to induce bone formation when implanted into animals.

5 It is an object of the invention to provide a method for the preparation of osteogenic protein from mammalian bone tissue in high yield. Another object is to provide bone-inducing devices comprising osteogenic protein adsorbed onto collagenous matrix as a delivery system for said bone morphogenetic proteins.

10 The invention provides osteogenic devices which, when implanted at a skeletal defect site of the mammal, induce at the site of implantation the full regeneration of bone and the consequent healing of the defect. The device comprises a collagenous matrix carrier material, as described below, and osteogenic protein, a fraction of total extractable bone protein which contains bone morphogenetic proteins (BMPs).

15 Osteogenic protein requires the presence of a suitable delivery material to exert its bone regenerating effects. Collagenous matrix purified from demineralised bone matrix is such a suitable material and is described in more detail below.

20 The method used to isolate osteogenic protein exploits in part, the published procedure of Sampath et al. (1987) (Proc. Natl. Acad. Sci. USA 84, 7109 - 7113. This procedure exploits the BMPs' affinity for heparin and hydroxyapatite immobilized onto chromatographic support matrices to achieve isolation of BMP rich fractions. The procedure entails the chromatography of urea  
25 extracts of demineralised bone onto a heparin chromatography column, followed by a hydroxyapatite column, and finally gel exclusion chromatography to eliminate heavy molecular weight contaminants. Although this procedure results in the effective isolation of a fraction with osteogenic capacity, the quantity, yield and speed of purification of osteogenic protein using the method of the present  
30 invention is greatly improved.

The preparation of the osteogenic protein of the invention is based on the procedure of Sampath *et al* (1987), but includes a novel and inventive modification. The key modification involves the fractionation of the total bone protein extract into a high and a low molecular weight fraction at the beginning of the purification process, before chromatography. The bone morphogenetic proteins have a molecular weight of approximately 30 kDa and, for the purposes of this specification, are classed as low molecular weight polypeptides. For the purposes of this specification, high molecular weight polypeptides include polypeptides with a molecular weight greater than 100 kDa and especially greater than 300 kDa. The high molecular weight fraction will include collagen and collagen fragments (approximately 100 kDa) as well as collagen aggregates (200 kDa and greater) and other unidentified polypeptides, some of which are thought to be inhibitors of morphogen-induced osteogenesis. It is important to note, for the purposes of this specification, that collagens are separated from the low molecular weight fraction at the beginning of the process before the heparin affinity chromatography step.

The removal of collagen is important for the following reasons. Firstly, collagen type I is known to have an affinity for BMPs (Reddi AH (1995) Cartilage morphogenesis: role of bone and cartilage morphogenetic proteins, homeobox genes and extracellular matrix. Matrix Biol. Oct 14(8):599-606. ; Winn SR, Uludag H, Hollinger JO. (1999) Carrier systems for bone morphogenetic proteins. Clin Orthop 1999 Oct (367 Suppl):S95-106). Secondly, collagenous peptides are large MW peptides which tend to foul columns and alter the exchange dynamics of the BMP with the binding sites on the heparin molecule in a way which hampers binding. Furthermore, it appears that there may exist inhibitors of bone morphogenetic proteins, the active constituent of osteogenic protein, that reside in the high molecular weight fraction of total extractable bone protein. This implies that there exists competitive binding for BMPs between collagen type I and heparin. This competitive binding interference appears to result in yield losses during the chromatographic purification of BMPs on a heparin column. The method of this invention results in a significant improvement in the recovery of total osteogenic activity over prior art methods.

The invention is now described, by way of example with reference to the following Example and the Figure in which

Figure 1 shows X-ray evaluation scores of treated non-unions as a function of time;

Figure 2 shows non-union in a bone of a patient after conventional treatment; and

Figure 3 shows complete healing of the bone of the patient of Figure 2 after treatment in accordance with the method of the invention

### EXAMPLE 1

#### **Purification of human osteogenic protein containing bone morphogenetic proteins**

##### **1. Preparation of Demineralised Bone**

Human long bone diaphyses, freed from adhering soft tissues, were demarrowed and cut into pieces of between 1 and 4 cm. Batches of this material were defatted in a solvent system comprising a 50:50 ratio by volume of methanol and chloroform, at 4°C - 8°C for 16 - 24 hours. The bone was then dehydrated in absolute alcohol for 48 hours at 4°C - 8°C. The alcohol was decanted and the bone was air-dried in a fume hood for 48 - 96 hours. The bone was then milled in a hammer mill to a particle size ranging from 10 to 425 micron.

The particulate material was demineralised at room temperature, with consecutive additions of four to five volumes of 0.5 M HCl, until acid base reaction between the hydroxyapatite of the bone and the HCl had neared completion as judged by slowing pH changes over time. The demineralised bone was neutralized with dilute sodium bicarbonate solution, and washed with purified water to produce demineralised bone matrix.

## 2. Dissociative extraction of Demineralised Bone Matrix

Demineralised bone matrix (DBM) from the previous step was extracted twice with three to four volumes of 8M urea, 50mM Tris-HCl, pH 7.4, containing protease inhibitors (5 mM benzamidine hydrochloride, 0.1 M 6-aminohexanoic acid, 5 mM N-ethylmaleimide and 0.5 mM phenylmethylsulfonylfluoride) for 24 hours at 4°C to 8°C. The supernatant was collected by filtration through a porous polypropylene frit, and filtered through a three micron nominal size cartridge filter (Polygard, Millipore Corporation, USA).

## 3. Ultrafiltration fractionation of high molecular weight components

Heavy molecular proteins and collagens were removed by ultrafiltration of the supernatant from the step 2 through a polysulfone 300 kDa nominal molecular weight membrane (Millipore, Cat. No. CDUF006TM). A 100 kDa nominal molecular weight membrane can optionally be employed with somewhat lower yields of total BMP activity, but with higher specific activity. This procedure removed collagens, especially type I collagens, which bind BMPs under conditions of lower ionic strength. The retentate was washed a few times with 6 M urea buffer, 50 mM Tris-HCL pH 7.4 (Buffer A), and the diafiltrate which contained the osteogenic activity was collected.

## 4. Concentration and desalting by Ultrafiltration buffer exchange

The diafiltrate containing the osteogenic proteins (molecular weight circa 30 kDa) from step 3 was desalted and concentrated by ultrafiltration on a 10 kDa PLGC membrane (Millipore, Cat. No. SK1P003W4). This step effectively removed salt and other low MW weight components, to create the required conditions for the following chromatographic step. Successive volumes of Buffer A containing the aforementioned concentrations of enzyme inhibitors but excluding n-ethyl maleimide were added to the retentate following concentration, until the conductivity of the retentate reached between 5.0 and 6.0 milli Siemens.

5. Heparin-Sepharose chromatography

The retentate from step 4 was chromatographed onto Heparin-Sepharose CL-6b (Pharmacia-Amersham) which had been equilibrated with buffer A containing 0.15 M NaCl. The column was washed with three column volumes of buffer A containing 0.15 M NaCl and then eluted with buffer A containing 0.5 M NaCl. The eluting peak with absorbance at 280 nm was collected and stored at 4°C.

6. Ultrafiltration exchange of heparin-Sepharose affinity fraction

The Heparin-Sepharose affinity fraction from step 5 was desalted and concentrated by ultrafiltration on a PLCC 5 kDa membrane (Millipore, Cat. No. CDUF001LC). This step effectively removed salt and other low MW weight components, to create the required conditions for the next chromatographic step. Successive volumes of Buffer A containing 10 mM sodium phosphate were added to the retentate following concentration, until the conductivity of the retentate had reached between 5.0 and 6.0 milli Siemens.

7. Hydroxyapatite (HA) chromatography

The retentate from step 6 was chromatographed onto a hydroxyapatite column (Hydroxyapatite Ultrogel, Biosepra, France) which had been equilibrated in Buffer A containing 10 mM sodium phosphate. The column was washed with three column volumes of Buffer A containing 10 mM sodium phosphate. An osteogenic protein enriched fraction was eluted with Buffer A containing 150 mM sodium phosphate. The eluting peak with absorbance at 280 nm was collected and stored at 4°C.

8. Exchange of HA affinity fraction into 10 mM HCl

The HA affinity fraction from step 7 was exchanged into a 10 mM HCl solution using an Amicon stirred Ultrafiltration cell (Millipore Corporation, U.S.A.)

loaded with a 3 kDa cutoff cellulose membrane (YM3, 76 mm regenerated cellulose, Millipore Corporation U.S.A.).

In an embodiment of the invention, the HA affinity fraction was instead loaded onto a C-18 Vydac silica-based HPLC column (particle size 5  $\mu$ m, pore size 300 Å). The column was washed with 0.1% trifluoroacetic acid, 10% acetonitrile for 10 column volumes, and the bound proteins were step eluted with a 70% acetonitrile, 0.1% trifluoroacetic acid. This material was lyophilized and reconstituted into 10 mM HCl.

The process flow chart with protein values is set out in Table 1.

TABLE 1.

| Step | Procedure  | Total protein (mg) |
|------|--|--------------------|
| 1    | Dehydrated, defatted human bone powder 5 kg.   | Not determined     |
| 2    | Extraction with chaotropic solution containing urea or guanidinium chloride  | 17 703             |
| 3    | Filtration through a Millipore Polygard CR cartridge filter with a 3.0 micron nominal pore size product no. CR0301006. | Not determined     |
| 4    | Ultrafiltration employing a membrane with a nominal pore size of 300 kD  | 15 069             |
| 5    | Ultrafiltration and buffer exchange employing a membrane with a nominal pore size of 10 kD                             | 8 881              |
| 6    | Heparin affinity fraction  | 211.43             |
| 7    | Hydroxyapatite affinity fraction   | 62.99              |
| 8    | Ultrafiltration exchange or HPLC   | 40-60              |

500 $\mu$ g of the material from step 8 of Example 1, delivered on 1.2 grams of collagenous matrix, induces new bone formation in recalcitrant long-bone non-unions in humans. The material from step 8 was analysed by S-200 gel

filtration chromatography (Pharmacia) and found to contain 20% by mass of high molecular weight components. These may be optionally removed by a 'polishing' step employing gel exclusion chromatography on S-200 matrix (Pharmacia) and elution with 8M Urea, 1M NaCl, 50mM Tris-HCl pH 7.4. The final yield is in the region of 30 mg to 50 mg of osteogenic protein. These yields are approximately four-fold higher than those previously reported for baboon bone extraction employing a comparative starting bone material and method on hydroxyapatite affinity, heparin affinity and gel exclusion chromatography on S-200 matrices (Pharmacia)(Ripamonti U, Ma SS, Cunningham NS, Yeates L, Reddi AH (1993) Reconstruction of the bone-bone marrow organ by osteogenin, a bone morphogenetic protein, and demineralised bone matrix in calvarial defects of adult primates Plast Reconstr Surg 91(1):27-36). Comparative histomorphometric studies between iliac crest bone biopsies of humans and baboons have demonstrated a remarkable degree of similarity between the two species (Schnitzler CM, Ripamonti U, and Mesquita JM (1993) Histomorphometry of iliac crest trabecular bone in adult male baboons in captivity, Calcif. Tiss. Int., 52, 447 - 454).

## EXAMPLE 2

### **Determination of osteogenic activity of osteogenic protein**

#### Bioassay in rats

Osteogenic activity was bioassayed as described by Sampath and Reddi (Proc. Natl. Acad. Sci. USA (1983) 80:6591-6595). The assay consists of implanting test samples comprising allogeneic insoluble collagenous bone matrix and human osteogenic protein in subcutaneous sites in recipient rats under ether anesthesia. A vertical incision (1 cm) was made under sterile conditions in the skin over the thoracic region, and bilateral pockets were prepared by blunt dissection. Implants comprised 25 mg rat ICBM, 50 mg rat tail type I collagen in 0.5 M acetic acid, and osteogenic protein in varying amounts. The test sample was implanted bilaterally into each pocket and the incision was closed with stitches. The heterotropic site allowed for the study of bone induction without the possible ambiguities resulting from the use of bony sites.

Regenerated tissues were explanted on day 12 post-implantation and assayed for alkaline phosphatase activity, a marker for bone formation, as described (Reddi and Sullivan 1980 Endocrinology 107:1291 – 1299). Results and data are presented in Table 2.

TABLE 2

| MATERIAL           | AMOUNT  |
|--------------------|---------|
| Osteogenic protein | 500 g   |
| HICBM*             | 1000 mg |
| Human gelatin      | 200 mg  |
| TOTAL              | 1200 mg |

\*hICBM- human insoluble collagenous bone matrix.

The implant model in rats exhibited a controlled progression through the stages of osteogenic protein induced endochondral bone development. This postfoetal osteogenesis may be considered to recapitulate events that occur in the normal course of embryonic bone development. The new bone resulted from local mesenchymal condensations, a cartilage phase and extracellular matrix production, vascular invasion and mineralisation, and finally the formation of new bone *via* the differentiation of osteoprogenitor cell lines.

Histological analysis employing staining with toluidine blue or hemotoxylin/eosin demonstrated clearly the development of endochondrial bone. Twelve day implants were usually sufficient to determine whether the implants showed bone inducing activity.

Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization of the implant and assaying of enzyme activity with the substrate p-nitrophenyl phosphate under alkaline conditions. Implants showing no bone development by histology should have no alkaline phosphatase activity under these assay conditions (Reddi AH and Sullivan NE (1980) Endocrinology 107, 1291 – 1299). The assay is useful for quantitation of the specific and total activity of alkaline phosphatase, which may then be correlated to the osteoinductive potency of the prepared osteogenic protein described herein.

Alkaline phosphatase activity is calculated according to the method of Reddi and Sullivan (1980, Endocrinology 107, 1291-1299). Induction of 1 unit or more of alkaline phosphatase by a rat implant indicates effective osteogenesis.

### EXAMPLE 3

#### **Preparation of Human gelatin**

An amount of ICBM was combined with five to ten volumes of water in a borosilicate glass bottle and heated in a pressure cooker for one hour. The supernatant was filtered through Whatman no. 1 paper or a 20 micron stainless steel mesh. The gelatinous solution was cooled to 25 °C and 5 volumes of chilled ethanol (-20° C) were added to precipitate collagen. The precipitate was dried *in vacuo*, and milled to a size range of 75 to 425 micron.

### EXAMPLE 4

#### **Fabrication of Osteogenic Devices**

Human ICBM was used as the adsorptive carrier matrix for the fabrication of the osteoinductive composite biomaterial. Inactive ICBM was restored to biological activity when a sufficient amount of osteogenic protein was combined with ICBM. The particle size of the ICBM influences the quantitative response of new bone.

Particles between 75 and 420  $\mu\text{m}$  elicit the maximum response. An amount of ICBM was combined with osteogenic protein in 10 mM HCl and thoroughly mixed with sterile spatula. The material was lyophilized to dryness.

5 This material was then combined with human ICBM-derived gelatin. The components were thoroughly dry-mixed together to obtain a homogeneously distributed composition. The human gelatin acted as a readily hydratable material that causes the biomaterial to become extrudable. The composite was packed into a syringe. The osteoinductive composite was rehydrated at the time of use. Rehydration was achieved when an amount of sterile saline or water was drawn  
10 into the syringe, and approximately 10 minutes allowed for rehydration to occur. The material could then easily be expelled out of the syringe by depressing the plunger. This allowed for precise implant deposition into a defect site at the time of surgery. The implant may be further contained *in situ* using standard gelatinous sponges such as Spongostan (Johnson and Johnson Medical Limited, U.K.).

15 The carrier could be replaced by either a biodegradable-synthetic or synthetic-inorganic matrix (e.g., HAP, collagen, tricalcium phosphate, or polylactic acid, polyglycolic acid and various copolymers thereof).

20 Table 3 sets out a typical formulation for the osteogenic composition.

TABLE 3

| Material           | Amount  |
|--------------------|---------|
| Osteogenic protein | 500 µg  |
| hICBM*             | 1000 mg |
| Human gelatin      | 200 mg  |
| TOTAL              | 1200 mg |

\*hICBM- human insoluble collagenous bone matrix.

### EXAMPLE 5

#### Testing of Osteogenic composition in humans

10      Implants containing 500 micrograms of human osteogenic protein adsorbed onto a  
 composite matrix comprising 1g of insoluble collagenous bone matrix and 200 mg  
 of lyophilized human gelatin were prepared. Thirty-four patients with resistant  
 nonunions including partial or complete segmental defects were treated with the  
 osteogenic composite. The series consisted of 11 females and 23 males. The  
 15      average age was 36 years. All patients had previously been variously treated by  
 internal or external fixation, cast, and/or allogeneic or autogeneic bone grafting,  
 and failed to achieve union. Preoperative symptoms averaged 26 months (range,  
 one to 228 months). The implant was incorporated at the time of surgery by  
 injecting the hydrated implant at the defect site, which was further stabilised by  
 20      internal or external fixation. An average of 2.4g of the composite was used per  
 patient. Seventeen patients additionally received supplementary allogeneic bone  
 which included allogeneic cancellous bone particles and block configured spongy

bone. Functional results were rated according to weight-bearing function at follow-up periods of 1, 8, 16 and 24 week periods post-operatively. A zero score was allocated where there was no weight bearing, a one score allocated for weight bearing with the assistance of two crutches, a two score allocated for light weight bearing with one crutch, a three score allocated for full weight bearing with one crutch and a four score allocated for full weight bearing with no crutch assistance. The average score was 3.25 for an average follow-up time of 17 weeks (range eight to 32 weeks). This score was higher than the pre-operative score of 2.22 and the post-operative score (week 1) of 0.5. Of the five patients who suffered recurrent infection, two failed to score above 2 at 18.5 weeks average follow-up period. Bridging was as assessed radiographically by trained clinicians according to a scale from 1 to 5 where 1 = non-union/no callus, 2 = callus present without bridging, 3 = moderate bridging, 4 = good bridging, 5 = complete union. The average score for the treatment group on follow-up period of 20 weeks was 2.80 in comparison to the pre-treatment score of 1.44. The results indicate that the osteogenic composite implant of the invention results in effective treatment of difficult nonunions. The scores as measured at different follow-up periods are presented in Figure 1.

It is an advantage of the invention that the multistep developmental cascade of bone induced by the osteogenic biomaterial composite of the invention includes binding of fibrin and fibronectin to the biomaterial, chemotaxis of cells, proliferation of fibroblasts, mesenchymal condensation, differentiation into

chondroblasts, chondrogenesis, vascular invasion, bone formation, remodeling, and bone marrow differentiation.

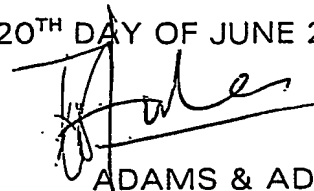
5 The injectable biomaterial of the invention offers several advantages. It may be stored at room temperature for lengthy periods without marked deterioration in biological activity. It may be readily rehydrated at the time of surgery and it is easily handled, merely requiring the depression of the syringe plunger to expel the osteogenic material as required.

10 From a clinical context, the osteogenic biomaterial composite offers the following advantages. It is osteogenic, inducing bone at the site of implantation. It obviates the need to perform a second operation at the patient's hip to harvest autologous bone and it obviates the need to use tissue banked bone. This reduces the risk of transmissible diseases.

15 The ICBM binds osteogenic protein and acts as a slow release delivery system to activate progenitor cells at the site of implantation. The composite biomaterial of the invention accommodates each step of the cellular response during bone development. It is biocompatible, and is resorbed during osteogenesis and replaced by the host's own bone.

20 The geometry of the described biomaterial as measured by its particle size, is optimal in permitting cell infiltration and differentiation.

DATED THIS 20<sup>TH</sup> DAY OF JUNE 2002



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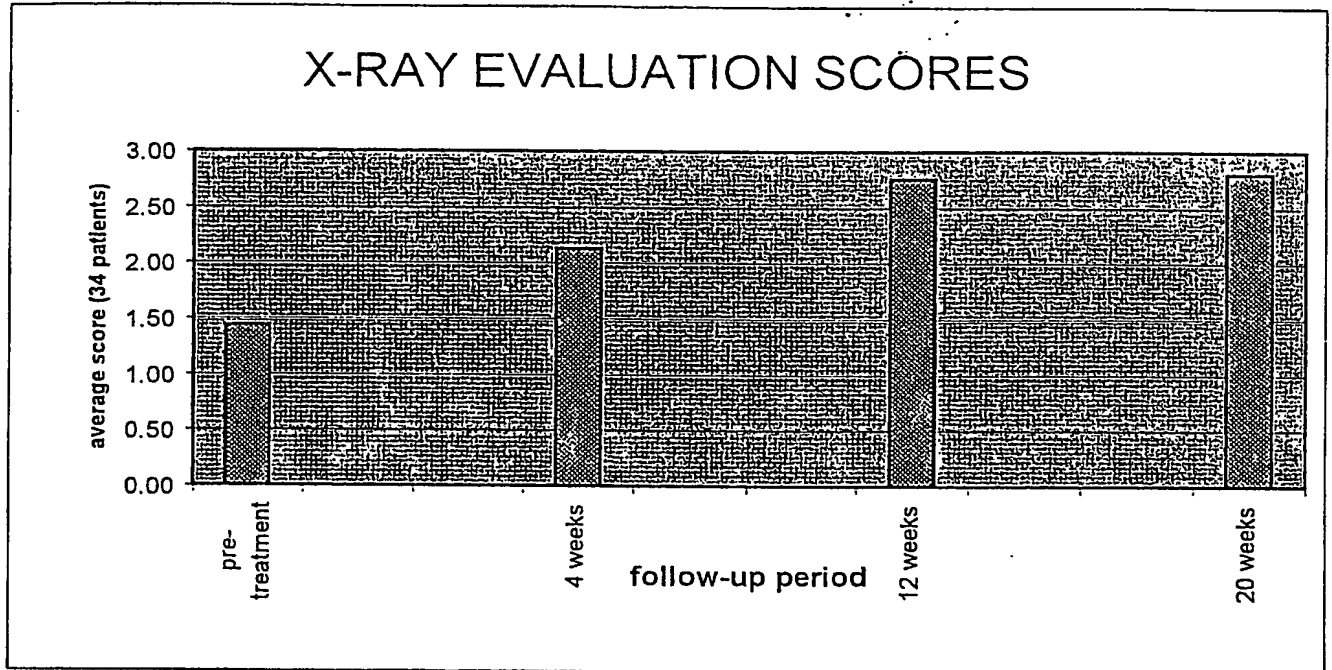


FIG. 1

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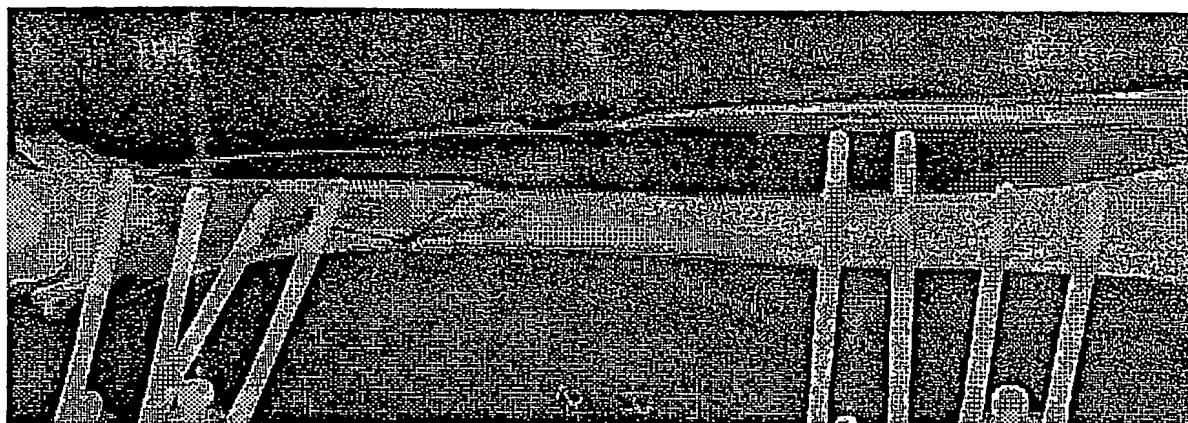


FIGURE 2. Non-union in a patient after attempts to treat defect conventionally had failed.

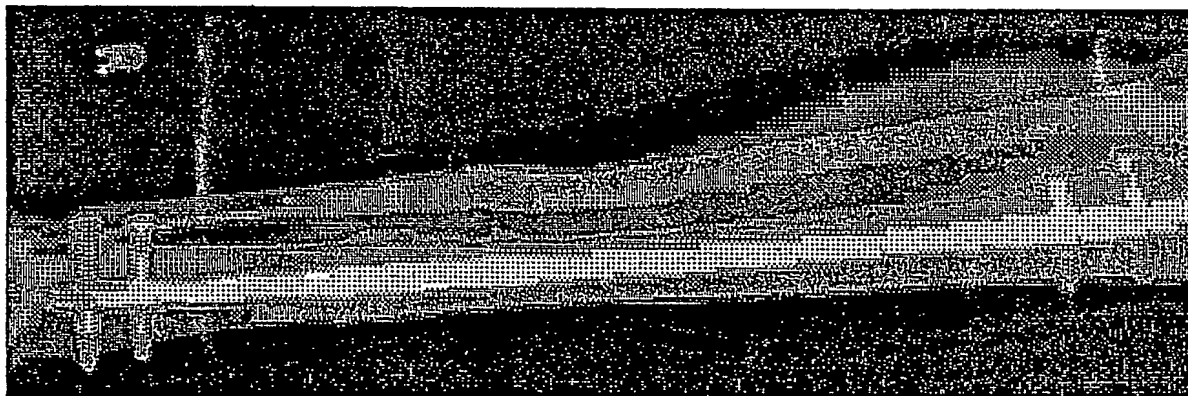


FIGURE 3. Same patient treated with the composite hBMP material and internal nail. Complete healing and visible new bone evidenced at 16 weeks post operatively.

*[Handwritten signature]*

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